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In vitro and in vivo Studies for Development of a Leishmaniasis Vaccine

FINAL REPORT

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The goal of this contract was to isolate antigens of Leishmania major and test them for their ability to impart protection against parasite challenge in mice. We preapred monoclonal antibody producing hybridomas from splenocytes of mice immunized with fixed promastigotes. We induced ascites production in mice given hybridomas ip, and purified antibody from ascites. The antigens recognized by selected monoclonal antibodies were assessed by immunoprecipitation and Western blot analysis. In addition, we developed a radioimmunoassay for detecting parasite antigens expressed on the surface of infected cells. We used this assay to screen monoclonal antibodies that recognized expressed strains.

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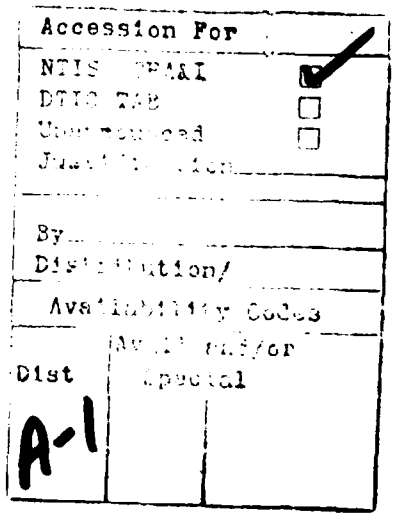
SUMMARY

The goal of the work supported by this contract was to identify antigens of leishmania that could serve as candidate vaccines against cutaneous leishmaniasis. During the eighteen months covered by this contract, we extended work begun under contract DAMD17-85-C-5015 toward this goal. We subcloned and expanded in vitro hybridomas that produced monoclonal antibodies against Leishmania major and produced antibody-containing ascites in mice injected intraperitoneally with hybridoma cells. On the basis of results of our initial efforts to characterize antigens recognized by the monoclonal antibodies using antibody-containing ascites, we decided that we needed to purify the antibody from the ascites. Since all the monoclonal antibodies were IgG_3 isotype, we could not employ the convenient method of staphylococcal protein A affinity purification. Instead, we tried a variety of ion exchange chromatography methods as well as two-step purification schemes (ion exchange followed by immunoaffinity transferrin depletion). These proved inadequate. Finally, we successfully purified IgG_3 monoclonal antibodies by a new method, streptococcal protein G affinity chromatography. This permitted us to begin analysis of antigens by Western blot methods using purified monoclonal antibody. In related studies, we unsuccessfully carried out immunoprecipitation assays of metabolically-labeled promastigote antigens. Our efforts to use this technique were stymied by the fact that labeled parasite material adsorbed non-specifically to plasma proteins including IgG. In as much as our interests were focused primarily on the leishmanial antigens expressed on the surface of infected macrophages, we developed a radioimmunoassay for detecting such antigens. While we were successful in detecting such antigens with polyclonal rabbit antibody, our results were less concrete using monoclonal antibodies. Unfortunately, the contract period was complete before we could try our purified monoclonal antibodies in this assay.



FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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REPORT

The ultimate goal of the work supported by this contract is development of a safe and effective vaccine against cutaneous leishmaniasis, for use by military personnel deployed to areas where the disease is transmitted. The motivation for this work resides in the need for and feasibility of such a vaccine. U.S. troops undergoing jungle warfare training in Panama and elsewhere have experienced a substantial risk of acquiring cutaneous leishmaniasis. Under battle conditions in endemic regions such as those in Latin America or the Middle East, disease acquisition can be expected to be at least as great as under controlled, simulated battle conditions. Insect repellants while potentially effective in reducing the risk have met with poor compliance by the soldier, even when utilized in the form of clothing-impregnated substances. No chemoprophylactic drugs are available.

The basis for optimism that a vaccine against cutaneous leishmaniasis could be developed lies in the following: 1) most individuals who recover from cutaneous leishmaniasis are resistant to rechallenge; 2) individuals given attenuated live parasites develop lesions that heal rapidly and are then rendered "immune", a practice carried out for centuries in Russia and the Middle East; 3) mice can be immunized against Leishmania major with partially-purified parasite material (1, 2).

A rational approach to leishmanial vaccine must take into consideration the overwhelming evidence that host defense in this disease is predominantly --if not exclusively-- cell-mediated (3,4). While in vitro treatment of promastigotes with antileishmanial monoclonal antibodies can incapacitate these organism's ability to induce infection in mice (5), there is no evidence that production of antileishmanial antibodies by infection or by immunization imparts any protection. The in vitro findings thus seem to represent an artefact as yet irreproducible in vivo; antibody transfer is not protective (6).

In vitro studies have indicated that the potential protective role of T cell mediated defenses in leishmaniasis resides their ability to secrete macrophage-activating factors such as interferon gamma (7,8) or by activating antileishmanial effects through cell-contact (9). As yet, there is no convincing evidence that cytotoxic lymphocytes play a role in this defense. Presumably, then, an effective vaccine would be required to induce such T cell defenses.

Vaccines have classically been developed with the idea that they stimulate antibody production, and indeed this notion directed the research on sporozoite vaccines against malaria (10). This notion and its application to vaccines probably is incorrect in some situations, however. On the otherhand, there are no well-defined vaccine strategies for inducing cell-mediated defenses, although such may be the consequence of emperic vaccine development efforts. Indeed, only clonotypic antibodies that represent internal images of T cell receptors have provided the first rational approach to a T cell vaccine (11).

The design of our studies is predicated on the notion that antigens retrieved using monoclonal antibody-based immunoaffinity chromatography will contain T cell epitopes that can induce T effector cells. We view as one requirement that the antigen be expressed on the surface of infected macrophages so that the requisite events that trigger lymphokine production or contact-mediated activation can occur (12). In other words, we consider the antigens of interest ("target antigens") as those displayed on the infected macrophage surface. Accordingly, the design of the study involves production of monoclonal antibodies to Leishmania as well as development of a reliable assay for screening their potential specific reactivity with the parasite antigens expressed on the surface of infected cells.

1. Background research

Prior to the inception of the present contract, we had begun to develop monoclonal antibodies and were attempting to devise an appropriate method for detecting antigens expressed on the surface of infected cells. The contract period covered by this report represents a continuation of that work, which was supported by contract DAMD 17-85-C-5015. The results of the earlier work were reported in detail in the final report of that contract. In as much as it has direct bearing on the work carried out under the present contract, the results will be briefly reviewed here.

We prepared monoclonal antibodies to L. major (NIH S strain [13]) by published methods (14). Briefly, promastigotes grown to stationary phase were fixed with gluteraldehyde and used with adjuvant to immunize mice. After boosting regimens were complete, splenocytes were fused with cells of the myeloma line P3X63Ag8 and hybrids were grown out in selective medium. Hybrids which secreted antileishmanial antibody (as detected by ELISA assay using fixed promastigotes) were

subjected to limiting dilution to establish clones. The ELISA assay entailed binding homologous promastigotes (10^6/well) to Immulon II plates with poly-L-lysine, adding hybridoma culture supernatants, and following washes, adding antimouse Ig (goat or rabbit-derived) conjugated with alkaline phosphatase. The rest of the assay was standard. The selected reactive monoclonal antibodies (culture supernatants) were also screened for reactivity with fetal bovine serum and human plasma fibronectin, in light of our observations that promastigotes adsorb serum proteins in vitro. The latter control proved appropriate; some monoclonal antibodies reacted with fibronectin and/or fetal bovine serum, and were discarded. This control should therefore be employed in all screening efforts involving promastigotes grown in the presence of mammalian proteins.

All our monoclonal antibodies from the first fusion were IgM isotype. This surprising finding was subsequently ascribed to the fact that a Sendai virus epidemic existed in our animal facilities during the immunization period. Since IgM is an inopportune isotype for use in immunoaffinity purification (posing special challenges to antibody purification), and since we were concerned that Sendai virus infection might somehow restrict the repetoire of antibody responses, we elected to carry out another fusion once our epidemic was under control.

The second immunization and fusion effort yielded several hybridoma clones that we subjected to at least two cycles of subcloning and rescreening. Again, some cloned hybridomas were discarded because they reacted with mammalian proteins. We finally expanded 11 clones/subclones. All proved to produce IgG_3 monoclonal antibodies. The present contract began while we were undertaking subcloning.

Concurrent with our efforts to develop monoclonal antibodies, we attempted to standardize a method for assessing expression of leishmanial antigens on the surface of infected cells. Prior investigations had suggested that such expression was indeed detectable by polyclonal (15) and monoclonal (16) antibody. We began by preparing rabbit polyclonal antileishmanial antibody (using homologous promastigotes as the immunogen). We used this antibody in an immunofluorescence assay whereby mouse peritoneal macrophages (infected or uninfected) were treated with antibody or pre-immune Ig, washed, and then treated with goat anti rabbit Ig conjugated with FITC. From these studies we determined that we needed a less subjective read-out system than visual assessment, and that we needed mononuclear phagocyte populations that were more homogeneous than those we could

subjected to limiting dilution to establish clones. The ELISA assay entailed binding homologous promastigotes (10^6/well) to Immulon II plates with poly-L-lysine, adding hybridoma culture supernatants, and following washes, adding antimouse Ig (goat or rabbit-derived) conjugated with alkaline phosphatase. The rest of the assay was standard. The selected reactive monoclonal antibodies (culture supernatants) were also screened for reactivity with fetal bovine serum and human plasma fibronectin, in light of our observations that promastigotes adsorb serum proteins in vitro. The latter control proved appropriate; some monoclonal antibodies reacted with fibronectin and/or fetal bovine serum, and were discarded. This control should therefore be employed in all screening efforts involving promastigotes grown in the presence of mammalian proteins.

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harvest from mouse peritoneum. Accordingly, we set out to develop a radioimmunoassay that employed leishmania-infected $P388D_1$ cells. The inception of this work coincided with the beginning of the present contract.

2. Production and purification of monoclonal antibodies

Subcloning and screening of hybridomas producing IgG_3 monoclonal antibodies resulted in the identification of several stable clones/subclones. These were injected into pristane-primed mice and expanded intraperitoneally for production of antibody-containing ascites. Of 5 original clones, sixteen subclones were productive of ascites that contained antipromastigote antibody with titers ranging from 1/25 to 1/1600 as determined in the ELISA assay.(The titration endpoint was defined as OD_{409} = 2X the standard deviation above the mean of negative controls). We experienced greater variability existed than we had anticipated in the ascites production rate we experienced with different hybridomas and also with the same hybridoma inoculated into different mice. We attempted to optimize ascites production by assessing the influence of hybridoma cell number or growth phase. These experiments indicated that no identifiable parameter that we could manipulate influenced ascites production. Although this critical practical point is largely ignored in the literature, it appears from our personal inquiries that it is a widespread and vexing experience.

Our efforts to produce amounts of ascites that would permit antibody purification were further frustrated by an unfortunate miscalculation. We routinely stored ascites in the presence of phenylmethylsulfonylfluoride (PMSF; a serine esterase inhibitor) to reduce proteolysis of antibodies . A technician who prepared the samples for storage reasoned that a 10-fold greater amount of inhibitor would be preferable than what we had advised. The isopropanol used to dissolve PMSF for the stock solution reached sufficiently high concentration under this protocol that it precipitated the IgG. As a result, on retrieving the stored ascites, we found that all the antibody titers had fallen precipitously. We carried out efforts to dissolve the precipitated Ig, but without avail. Accordingly, we had to begin ascites production again from scratch.

The next challenge was purifying IgG_3 from ascites. The need for this endeavor was our impression that crude ascites contained proteins that interferred with our assays (see below), and our awareness that to ultimately carry out immunoaffinity purification of parasite antigens we had to use purified Ig. This became particularly clear when we confirmed

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that certain parasite components tend to bind nonspecifically to serum proteins. Since our monoclonal antibodies were all IgG_3, an isotype with little affinity for staphylococcal protein A, we could not employ this convenient, widely-used ligand for purification. In assessing the alternative approaches, we were attracted to an ion exchange matrix "AbX" (Baker Chemical Co.) because of its putative property of excellent resolution of IgG isotypes. Unfortunately, our experience did not confirm the manufacturer's claims. The IgG fractions we obtained, when analyzed by SDS-PAGE, contained substantial contamination with other proteins. Albumin and transferrin, the most highly represented contaminants, posed particular problems (see below).

To deplete ascites of these two major proteins, we carried out double fractionation procedures. We first subjected the ascites to an antitransferrin immunoaffinity "scrubber" column that we could confirm was effective in removing most of the detectable transferrin. The non-binding proteins (present in the fall-through) were then subjected to DEAE Affi Blue, an ion exchange matrix particularly suitable for removing albumin. Unfortunately, this procedure provided us with an unacceptably low yield of antibody. As other variations on this theme also failed, we became aware of a relatively new affinity purification system, appropriate for IgG_3. Streptococcal protein G has excellent affinity for mouse Ig of this isotype. Using protein G-Sepharose affinity purification we were able to retrieve good yield of antibody that, by SDS-PAGE, was free of detectable protein contamination. Unfortunately, successful purification was only achieved at the end of the contract work period and we were thus had little time to utilize the purified antibodies.

3. **Parasite antigen analysis**

We undertook analysis of antigens recognized by monoclonal antibodies using two standard methods: 1) immunoprecipitation of metabolically-labeled parasite extracts; and, 2) Western blot analysis. Since differences have been observed in the composition of log and stationary-phase promastigotes (17), we first analysed proteins of promastigotes from log- and stationary- phase cultures labeled by ^{35}S-methionine pulse. SDS-PAGE of lysates were subjected to autoradiography. No difference in the autoradiographic chromatograms of promastigotes from the two stages of culture were discernible.

In related studies, we grew promastigotes to stationary phase, washed them, and transferred them to medium specifically deficient in methionine. After labeling with ^{35}S-methionine for 4h, we subjected the

parasites to three cycles of freeze-thaw and brief sonication in the presence of a protease-inhibitor cocktail. Subsequent ultracentrifugation separated the membrane-containing pellet and the soluble (cytosolic) supernatant. The pellet was solubilized with Triton X100. Both the pellet ("lysate") and cytosolic ("sonicate") fractions were used in immunoprecipitation studies.

Lysates and sonicates were reacted with monoclonal antibody-containing ascites or normal mouse IgG, followed by rabbit antimouse Ig, followed by protein A-Sepharose. The pelleted beads were treated in Laemmli buffer with SDS and reducing agent, and the supernatant was subjected to SDS-PAGE; the gel was dried and exposed to x-ray film. The resulting autoradiographs revealed extensive non-specific adsorption of labeled parasite proteins by normal mouse IgG. Indeed, we could not clearly distinguish autoradiograms from immunoprecipitates with normal IgG and ascites. We therefore pre-adsorbed lysates with the complex containing normal mouse IgG, rabbit antimouse Ig, and protein A-Sepharose, and then subjected the non-adsorbed material to immunoprecipitation with monoclonal antibody-containing ascites. The results suggested that the monoclonal antibodies tested all recognized proteins with Mr≅ 63 kD. We also assessed one of the IgM monoclonal antibodies from our first fusion, and found that it too recognized an epitope on a protein of this size.

A 63 kD glycoprotein has been identified as the immunodominant protein conserved among many Leishmania species (18), and is thought to have important biological properties relevant to parasite survival ( ). We therefore attempted to determine whether the proteins recognized by the monoclonal antibodies were glycosylated. We metabolically labeled promastigotes with ^{14}C-glucosamine or ^{14}C-mannose and carried out immunoprecipitation with rabbit polyclonal antileishmanial antibody. We found that preimmune rabbit serum and rabbit antileishmania antiserum precipitated the same material, indicating that the glycosylated substances bound non-specifically. No better success was achieved with ascites in comparison to normal mouse IgG.

Once we were able to successfully purify IgG_3 from ascites by protein G affinity purification, we were able to use the monoclonal antibodies to confirm the Western blot results obtained with crude ascites. Unfortunately, time did not permit studies that would have addressed the question of whether the monoclonal antibodies reacted with the same or distinct epitopes on the antigen.

4. Detection of parasite antigens on infected cells

We developed a solid-phase radioimmunoassay to objectively assess binding of antibodies to the surface of infected cells. Based on our inconsistent preliminary results obtained using murine resident peritoneal macrophages. we chose P388D_1 cells as the host cell for these assays. This murine macrophage-like cell line has many properties of tissue macrophages (20), grows well in culture, and is readily infected with L.major . The virtue of these cells is that they provide more-or-less homogeneous populations, in contrast to cells obtained by peritoneal lavage. This uniformity provided one important element in standardizing the assay.

The basic protocol we employed developed from studies that utilized our rabbit polyclonal antileishmanial antibodies. Cells were infected with footpad-derived amastigotes of L.major, usually t multiplicities of infection of 1:1. Cells were incubated for 24-36h in suspension cultures using our published methods (21). Cells were then washed, permitted to attach to wells of flexible plastic 96 well culture plates, and were fixed with paraformaldehyde. Control cells were left uninfected but were otherwise treated in an identical manner. Fixed cells were incubated with the primary antibody, either polyclonal of rabbit origin or murine monoclonal (hybridoma culture supernatants or antibody-containing ascites). After incubation, cells were washed and reacted with iodinated protein A (for rabbit antibody assay) or with rabbit antimouse Ig followed by the iodinated ligand. In some experiments, we attempted to amplify the signal by interposing an additional antibody into the "sandwich". Following washing, wells were excised and counted in a gamma counter. Standard controls included testing of pre-immune rabbit Ig and normal mouse IgG as well as secondary or tertiary antibodies or iodinated ligand without the other soluble components.

Based on comparison of cpm, substantially more polyclonal antibody bound to infected than uninfected cells (at dilutions that ranged from undiluted to 1:10,000). Furthermore, pre-immune Ig bound minimally to infected cells at all dilutions tested. We therefore employed this assay to investigate the kinetics of antigen appearance on the surface of the infected cells. Antibody reacted with cells as early as 1h post infection and there was no significant differences in the cpm of wells containing infected cells that were incubated with antibody at 1,4,6,20 or 25h of infection. We therefore could not exclude the possibility that the antigens "expressed" on the surface of infected cells were ones that adsorbed to the host cell surface (including whole amastigotes) rather

than derived from the phagolysosomal compartment containing internalized parasites (and thus ones of greatest immunological interest in the context of the goal of this contract). Alternatively, the results of these kinetic studies could represent the combination of initial adsorption of antigens (or whole parasites), followed by interiorization with concommitent reappearance on the host cell membrane of the antigens of interest.

To assess these possibilities, we examined antibody binding to live or fixed cells incubated with amastigotes for various intervals. We reasoned, and confirmed by visual examination, that amastigotes would bind to but would not be internalized by fixed host cells. Accordingly, it should be possible to separate the two putative phases of antigen expression--pre interiorization and post exteriorization. Indeed, we found that cpm of wells containing fixed cells incubated with amastigotes remained constant over a 4h coincubation, whereas cpm actually decreased from 30 min to 4h in cultures that initially contained live host cells. We interpret these findings to suggest that the antigens detected on cells infected for 24h most likely represents exteriorized ones. Consistent with the notion was our finding that only two of 14 hybridoma cuture supernatants apparently recognized expressed antigen in this assay (cpm > mean cpm recorded with all hibridomas + 2SD), while all reacted with isolated whole amastigotes.

We subsequently tested monoclonal antibody-containing ascites in this assay. To our chagrin, it appeared that infected cells might adsorbed ascites proteins other than IgG leading to spurious results in our radioimmunoassay. We considered it highly likely that the commercially-prepared rabbit anti mouse Ig also had specificity for transferrin (since ion exchange purification of serum used by commercial outfits to immunize these rabbits doubtfully remove all traces of transferrin.) This prompted us to identify a purification scheme for monoclonal antibodies in ascites that would circumvent this problem. As discussed above, such a method was identified only late in our studies. At the conclusion of the contract period, we were able to test one purified monoclonal antibody in this assay, and compared this with results obtained with ascites. At high concentrations, the purified monoclonal antibody appeared to bind specifically to infected cells.

5. Conclusions

The work carried out in this contract progressed to a point where we believe we had identified at least one, and probably two monoclonal

antibodies that recognized an expressed antigen. Its potential relation to gp63 was suggested by Western blot analysis, but required confirmation using the purified glycoprotein. Had time permitted, we would have proceeded to antigen purification and anticipate that we could have begun vaccination trials in mice within 3 months. Since submitting the contract proposal, we became aware of the potential utility of antiidiotypic vaccines (22) that might have utility in this disease. We submitted a competing renewal application for this contract focused on this goal. Despite an enthusiastically positive scientific review, funds were not made available to continue our work. We have been informed that leishmaniasis is no longer considered by the U.S. Army to be a disease meriting the level of research priority it previously received.

Our inability to remain on our planned schedule in these studies resulted from unexpected technical problems that we encountered. These included: 1) poor ascites production that required large numbers of mice and time to circumvent; 2) accidental denaturation of antibodies in ascites due to the addition of excess isopropanol contained in the PMSF solution requiring renewed efforts to produce ascites; and 3) the requirements of obtaining highly purified monoclonal antibodies (especially for use in the radioimmunoassay) and our initial difficulties in identifying a rapid method to do so.

Since this work culminated in production of one or more "reagents", and did not in of itself reveal new publishable information, no publications were or are to be forthcoming. We consider this a great pity, since for lack of additional contract funds we have been forced to cease further work that would have led to publishable new information.

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